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Celecoxib potently inhibits TNFα-induced nuclear translocation and activation of NF-κB

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ABSTRACT

Celecoxib is a specific inhibitor of cyclooxygenase 2 (COX2). While it has been used for the treatment of chronic inflammatory conditions, including rheumatoid arthritis, its detailed anti-inflammatory mechanism has not been clarified. Here, we found that Celecoxib potently inhibited TNFα-induced transcriptional activity and DNA binding activity of NF-κB; however, Celecoxib had no effect on TNFα-induced IKK activation and degradation of IκBα and IκBβ, suggesting that it inhibited NF-κB activation via suppressing downstream of IKK activation and IκBs degradation. Interestingly, it was also found that Celecoxib abrogated TNFα-induced nuclear accumulation of the NF-κB p65 subunit. As a result, TNFα-induced expression of inflammatory cytokines, CXCL1/KC and CCL2 /MCP-1, was clearly inhibited by Celecoxib. On the other hand, Celecoxib had no effect on the TNFα-induced nuclear translocation of c-jun and activation of ERK, JNK, p38 and Akt. Taken together, these data indicate that Celecoxib specifically inhibits TNFα-induced NF-κB activation at the level of its nuclear translocation. This negative regulation of NF-κB activation by Celecoxib might be an important mechanism leading to its anti-inflammatory activity.

1. Introduction

Prostaglandins (PGs) are members of a group of lipid compounds that are derived enzymatically from fatty acids and are known as important inflammatory mediators [1]. PGs are synthesized by two different forms of cyclooxygenase, enzymes designated cyclooxygenase 1 (COX1) and cyclooxygenase 2 (COX2) [2]. COX1 is constitutively present in tissues such as the gastrointestinal tract and kidney and produces physiologically important PGs [3,4]. On the other hand, COX2 activity is dramatically increased in inflammatory states and leads to inflammation and pain [4]. Conventional NSAIDs

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Abbreviations: COX2, cyclooxygenase 2; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; IκBa, inhibitor of NF-κB; IKK, IκB kinase; IL-1, interleukin-1; Jak, Janus kinase; JNK, c-jun N-terminal kinase; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor κB; NSAIDs, non-steroidal anti-inflammatory drugs; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; RA, rheumatoid arthritis; RIP, receptor-interacting protein; TNFα, tumor necrosis factor alpha; TRAF2, TNF receptor associated factor 2.

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inhibit both forms of enzymes and it is believed that NSAIDs causes side effects such as gastrointestinal toxicity [5]. Thus, molecular-based targeting of the COX2 isoform has led to the development of COX2 selective inhibitors such as Celecoxib, which potently inhibit COX2-dependent inflammation while avoiding typical NSAID-associated side effects [6,7].

Celecoxib, the first COX2-selective inhibitor, has been clinically applied to the treatment of rheumatoid arthritis (RA) for the relief of inflammation and pain [7–10]. RA is a systemic autoinflammatory disorder manifested by aggressive synovitis that over time causes bone, tendon, and cartilage damage [11]. A previous study reported that excessive amounts of various cytokines, including TNFα, IL-1, IL-6, IL-15 and IL-18 were produced by rheumatoid synovium [12,13]. Recently, the pathogenic significance of TNFα in the symptoms of RA has been demonstrated by the clinical efficacy of TNFα blockade as treatment [14,15]. Thus, it is commonly suggested that RA is mediated by a proinflammatory cytokine network with TNFα at its apex.

TNFα participates in the inflammatory effect by inducing various inflammatory cytokines, including CCL2/MCP-1, CXCL1/KC and IL-6, through the activation of transcription factor, NF-κB [16–18]. Upon cell stimulation with TNFα, TNF receptor I (TNFRI) recruits a serine/threonine kinase, receptor-interacting protein (RIP) and an adapter protein TNF receptor associated factor 2 (TRAF2) [19–21]. Consequently, it activates the downstream IKK complex, which is composed of two catalytic subunits, IKKα and IKKβ and a regulatory subunit, IKKγ [22–25]. In unstimulated cells, NF-κB remains inactive in the cytoplasm through the association with inhibitor proteins of the IκB family. Activated IKKs phosphorylate IκBs, leading to their degradation and the subsequent activation of NF-κB [22–25].

In this study, we focused on the effect of Celecoxib on the TNFα signaling pathway. Interestingly, we observed that Celecoxib significantly inhibited TNFα-induced NF-κB activation by preventing the nuclear translocation of p65 NF-κB subunit. In addition to COX2 inhibition, this inhibition of NF-κB activation by Celecoxib might contribute to its anti-inflammatory effects.

2. Materials and methods

2.1. Antibodies and reagents

Mouse TNFα was purchased from PEPROTECH (Rocky Hill, NJ, USA). Antibodies recognizing p65, lamin, IκBα, IκBβ, TNFRI, IKKα, IKKβ, IKKγ, TRAF2, β-actin and PE-conjugated antibodies recognizing TNFRI or TNFRII were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies recognizing JNK, phosphorylated JNK at Thr^{183}/Tyr^{185}, ERK, phosphorylated ERK at Thr^{202}/Try^{204}, Akt, phosphorylated Akt at Ser^{473} were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against RIP was purchased from BD Transduction Laboratories (Lexington, NY, USA). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse polyclonal IgG antibodies were purchased from Dako (Glostrup, Denmark). Celecoxib was gifted from Pfizer Japan Inc. (Tokyo, Japan). DMSO (Nacalai tesque, Tokyo, Japan) was used as a control.

2.2. Cell culture

Murine fibroblast cell line, NIH-3T3, cells were grown in DMEM medium (Nissui, Tokyo, Japan) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (BioWest, France), 100 U/mL of penicillin G (Nacalai tesque), 100 μg/mL of streptomycin (Nacalai tesque) and 4 mM L-glutamine (Nacalai tesque). NIH-3T3 stably expressing the NF-κB-dependent luciferase reporter plasmid was established as described below. NIH-3T3 cells were transfected with 5 μg of pNF-κB Luc (Stratagene, CA, USA) and 0.5 μg of pBabePuro by Lipofectamine 2000 (Invitrogen, MD, USA). After 24-h incubation, the cells were cultured with DMEM containing 10% FBS and 7.5 μg/mL puromycin (Nacalai tesque) for 2 weeks. Puromycin-resistant colonies were picked up and grown in selection medium continuously. Each clone was evaluated by assay testing TNFα-induced luciferase induction ability. The most responsible clone (KF8) was used in the following experiments.

2.3. NF-κB luciferase assay

KF8 cells (5×10^4 cells) were cultured in a 24-well plate and preincubated with various concentrations of Celecoxib for 1 h at 37 °C. After treatment with TNFα (10 ng/mL) for 6 h, the cells were harvested and lysed in passive lysis buffer (Promega, Madison, WI, USA). Luciferase activity of the lysates was determined using the luciferase reporter assay system (Promega), according to the manufacturer's instructions. NF-κB-dependent luciferase activity was normalized by the quantity of protein for each sample.

2.4. Cell viability analysis

Cells (5×10^4 cells) were preincubated with various concentrations of Celecoxib for 1 h at 37 °C. After treatment with TNFα (10 ng/mL) for 6 h, the cells were collected by trypsinization, and were then analyzed by trypan blue exclusion tests using a cell viability analyzer (Beckman Coulter Inc., CA, USA).

2.5. Immunofluorescence assay

Cells (5×10^5 cells) were seeded on sterile coverslips and pretreated with Celecoxib (25 μM) for 1 h following stimulation with TNFα for 30 min. After washing with PBS (Nacalai tesque) three times, the cells were fixed in 4% paraformaldehyde (Nacalai tesque) for 10 min at room temperature and washed with PBS three times. Cells on coverslips were permeabilized in 0.2% (v/v) Triton X-100 (Nacalai tesque) for 5 min at room temperature. After washing with PBS three times, the coverslips were blocked in PBS containing 3% FBS for 5 min and incubated with an antibody recognizing p65 (Santa Cruz Biotechnology) diluted with PBS containing 3% FBS at 1:200 dilution for 1 h at room temperature. After washing with PBS three times, the coverslips were incubated with a secondary antibody BODIPY FL® anti-rabbit IgG (Invitrogen) at 1:200 dilution for 1 h at room temperature. After washing with PBS three times, the coverslips were mounted using VECTA SHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Each coverslip was analyzed on an

OLYMPUS BX50 microscope (Olympus, Tokyo, Japan) with Olympus Micro DP70 software (Olympus).

2.6. Flow cytometry analysis

Cells (1×10^6 cells) were preincubated with Celecoxib (25 μM) for 1 h at 37 °C. After treatment of TNFα (10 ng/mL) for 15 min, the cells were washed in PBS containing 0.5% bovine serum albumin (BSA) (Nacalai tesque) and reacted with PE-conjugated antibodies recognizing TNFRI or TNFRII (Santa Cruz Biotechnology) on ice for 1 h. Cells were washed in PBS containing 0.5% BSA, suspended in 200 μL of PBS and then analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Cowley, United Kingdom) and analyzed using Cell Quest software (Becton Dickinson) as described previously [26].

2.7. Immunoblot analysis

Nuclear and cytoplasmic extractions were performed as reported [27]. Cells were washed with PBS and lysed in lysis buffer containing 50 mM Hepes, pH 7.5, 0.5% Triton X-100, 100 mM NaF, 10 mM sodium phosphate, 4 mM EDTA, 2 mM Na_3VO_4, 2 μg/mL aprotinin and 2 μg/mL leupeptin (Nacalai tesque). Cell lysates were centrifuged at 15,000 rpm, 4 °C for 15 min to remove the debris and the protein concentration was determined by Bradford assay. Eluted proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were probed by using the designated antibodies and visualized with the ECL detection system (GE Healthcare UK Ltd., Buckinghamshire, United Kingdom) as described previously [26].

2.8. In vitro IKK assay

Cells (1×10^7 cells) were preincubated with Celecoxib (25 μM) for 1 h at 37 °C following stimulation of TNFα (10 ng/mL) for the indicated times. Cell lysates were immunoprecipitated with anti-IKKγ antibody and Protein G Sepharose (Zymed, South San Francisco, CA, USA) for 2 h at 4 °C and then washed three times with lysis buffer and twice with kinase buffer (25 mM Hepes–NaOH (pH 7.5), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM dithiothreitol and 20 mM *p*-nitrophenylphosphate (Nacalai tesque)). The kinase reaction in 20 μL of kinase buffer including [γ-^{32}P] ATP was carried out with 1 μg of GST-IκBα as a substrate for 20 min at 30 °C. Samples were resolved by SDS-PAGE and phosphorylated GST-IκBα was visualized by autoradiography, as described previously [26].

2.9. RNA isolation and real-time PCR

RNA was prepared using an RNA purification kit (Qiagen, Hilden, Germany). RT was performed using an oligo$(dT)_{20}$ primer and 1 μg total RNA for first-strand cDNA synthesis, as described previously [26]. Quantitative real-time PCR was performed using an ABI Prism 7900HT Sequence detection system (Applied Biosystems, Scoresby Vic, Australia). Primer sequences were as follows: GAPDH, 5′-ACTCCATCCG-CAATTC-3′ (upstream) and 5′-CCTTCCACAATGCCAAAGTT-3′ (downstream); CXCL1/KC, 5′-TGGGGACACCTTTTAGCATC-3′ (upstream) and 5′-GCCCATCGCCAATGAG CTG-3′ (downstream); CCL2/MCP-1, 5′-TGAGGTGGTTGTGGAAAAGG-3′ (upstream) and 5′-CCTGCTGTTCACAGTTGCC-3′ (downstream).

2.10. Electrophoretic mobility shift assay (EMSA)

Consensus double-strand oligo-deoxynucleotide probes for NF-κB (TAGTTGAGGGGACTTTCCCAGGC) were radioactively labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase (TAKARA, Shiga, Japan) as described previously [26]. Then, 2 μg of nuclear extract was incubated with a γ-^{32}P-labeled double-stranded oligonucleotide probe in buffer containing 10 mM Hepes–KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 20% glycerol, 25 mM dithiothreitol, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 5 mM Na_3VO_4 (Nacalai tesque). The binding reaction was carried out at 30 °C for 20 min in a total volume of 25 μL. Bound complexes were separated by 4% polyacrylamide gel electrophoresis in Tris–glycine–EDTA (TGE) buffer and visualized by autoradiography.

2.11. Enzyme-linked imunosorbent assay (ELISA)

Cells (5×10^4 cells) were pretreated with Celecoxib (25 μM) for 1 h at 37 °C. After treatment with TNFα (10 ng/mL) for 24 h, the supernatants were harvested and the amounts of CXCL1/KC and CCL2/MCP-1 were determined using immunoassay kits (R&D Systems, Minneapolis, MN, USA).

3. Results

3.1. Celecoxib inhibited TNFα-induced NF-κB activation in a dose-dependent manner

Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl] benzenesulfon amide) is a non-steroidal anti-inflammatory drug that is a specific inhibitor of COX2 (Fig. 1A). In order to investigate the anti-inflammatory effect of Celocoxib, we evaluated the effect of Celecoxib on NF-κB activation. KF 8 cells, which stably express the NF-κB luciferase reporter gene, were treated with various concentrations of Celecoxib for 1 h following TNFα stimulation. As shown in Fig. 1A, TNFα-induced NF-κB activation up to about 20 times compared with an unstimulated cell. Interestingly, Celecoxib potently inhibited TNFα-induced NF-κB activation in a dose-dependent manner with IC_{50} = 22.5 μM (Fig. 1B). On the other hand, DMSO itself had no effect on TNFα-induced NF-κB activation. As shown in Fig. 1C, cell viability was not changed by the treatment of Celocoxib and/or TNFα stimulation, thus indicating that the inhibition of NF-κB activation by Celecoxib is not attributable to its cytotoxity.

3.2. Celecoxib had no effect on the expression of signaling molecules in the TNFα signaling pathway

In order to determine how Celecoxib inhibited NF-κB activation induced by TNFα, we investigated its effect on the expression levels of major signaling molecules which are

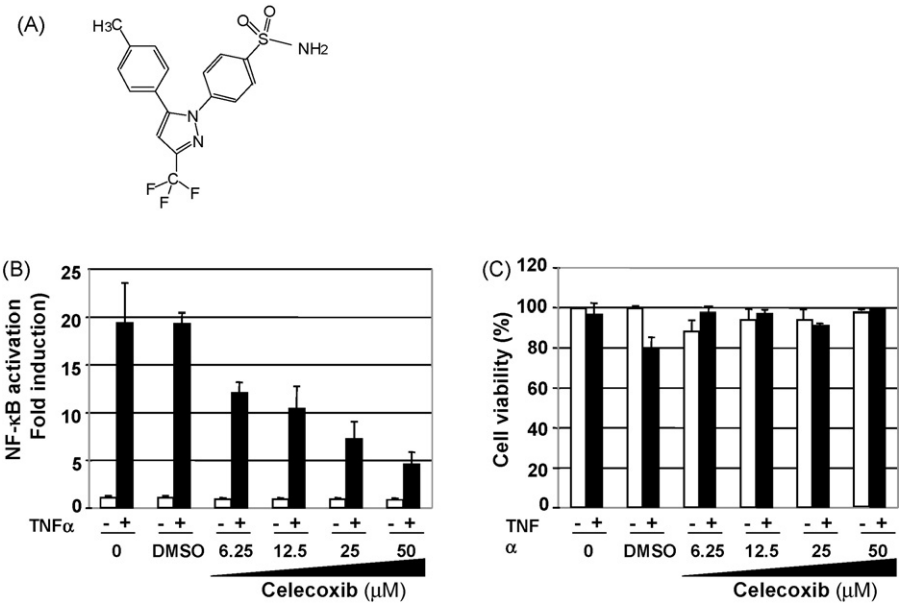

Fig. 1 – Celecoxib significantly inhibited TNFα-induced NF-κB activation. (A) Structure of Celecoxib. (B) KF8 cells were pretreated with different concentrations of Celecoxib or DMSO (0.1%) for 1 h following stimulation with TNFα (10 ng/mL) for 6 h. NF-κB-dependent luciferase activity was normalized to the protein amounts. Data are the mean ± S.D. of the relative luciferase activities of pNF-κB-Luc in four experiments. (C) KF8 cells were pretreated with different concentrations of Celecoxib or DMSO for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for 6 h. Cell viability was examined with trypan blue exclusion tests.

required for the TNFα signaling pathway. First, we analyzed the expression of the mature form of TNFRI and TNFRII on the cell surface by flow cytometry analysis. As shown in Fig. 2A, similar levels of cell-surface TNFRI and TNFRII were observed in untreated cells and treated cells with Celecoxib. Interestingly, the cell-surface expression levels of these receptors were down-regulated by TNFα stimulation; however, Celecoxib had no effect on these down-regulations. Upon TNFα stimulation, a serine/threonine kinase, RIP, and an adaptor molecule, TRAF2, were recruited to TNFRI [19–21]. Consequently, NF-κB is rapidly activated through the activation of IKK complex [22–25]; however, the expression levels of RIP, TRAF2, IKKα, IKKβ and IKKγ were not changed in cells treated with 20 μM of Celecoxib in the absence and presence of TNFα stimulation (Fig. 2B). Therefore, these data confirmed that the inhibition of NF-κB activation by Celecoxib was not due to the altered expression of signaling molecules in the TNFα signaling pathway.

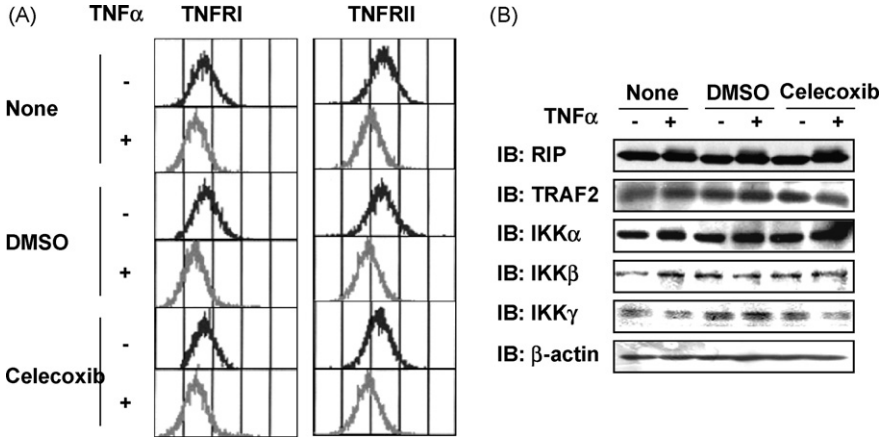

Fig. 2 – Celecoxib had no effect on the expression of signaling molecules in TNFα signaling pathway. KF8 cells were pretreated with Celecoxib (25 μM) or DMSO (0.1%) for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for 15 min. (A) Cell surface expressions of TNFRI and TNFRII were assessed by flow cytometry analysis. (B) Whole cell lysates were immunoblotted with antibodies recognizing RIP, TRAF2, IKKα, IKKβ, IKKγ or β-actin.

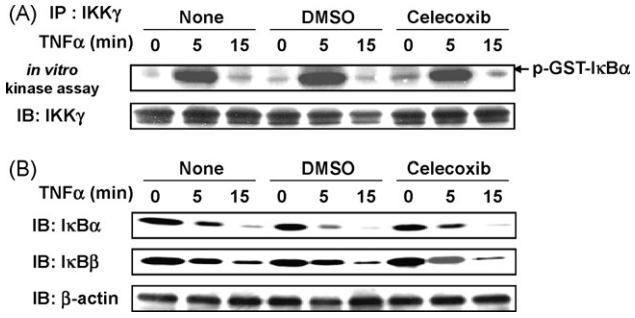

Fig. 3 – Celecoxib had no effect on TNFα-induced IKK activation. (A) KF8 cells were pretreated with Celecoxib (25 μM) or DMSO (0.1%) for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for the indicated periods. Cell lysates were immunoprecipitated with anti-IKKγ antibody. IKKγ immunoprecipitates were assayed for kinase activity using purified GST-IκBα as a substrate (upper). Immunoprecipitates were immunoblotted with anti-IKKγ antibody (lower). (B) KF8 cells were pretreated with Celecoxib (25 μM) or DMSO for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for the indicated periods. Whole cell lysate were immunoblotted with antibodies against IκBα, IκBβ or β-actin (as a control).

3.3. *Celecoxib did not inhibit TNFα-induced IKK activation*

Since the activation of IKK is a key step in the activation of NF-κB [22–25], we next determined whether Celecoxib inhibits TNFα-induced IKK activation by *in vitro* kinase assay. IKK complex was immunoprecipitated with anti-IKKγ antibody and IKK activity was measured as IκBα phosphorylation using GST-IκBα. As shown in Fig. 3A, TNFα-induced IKK activation; however, Celecoxib and DMSO had little effect on IKK activation. Furthermore, when the degradation of IκBα and IκBβ after TNFα stimulation was examined by Western blotting, TNFα induced the degradation of IκBα and IκBβ in 5 min and Celecoxib did not inhibit the degradation of IκBs in response to TNFα (Fig. 3B). Thus, these data indicate that Celecoxib inhibited NF-κB activation via suppressing downstream of IKK activation.

3.4. *Celecoxib inhibited the TNFα-induced nuclear translocation of NF-κB*

Next, we investigated the effect of Celecoxib on the TNFα-induced nuclear translocation of NF-κB using cytoplasmic and nuclear extracts. As shown in Fig. 4A, TNFα induced the accumulation of the p65 NF-κB subunit in the nucleus in 5 min. Strikingly, Celecoxib decreased the amount of nuclear NF-κB

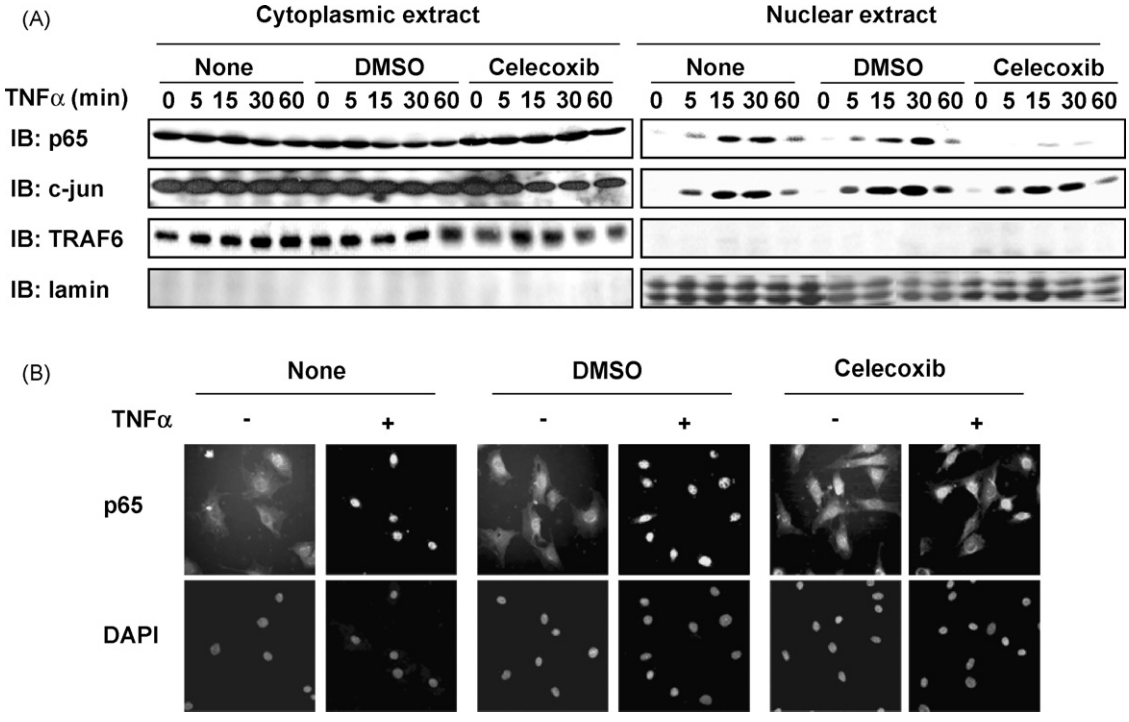

Fig. 4 – Celecoxib specifically inhibited TNFα-induced nuclear translocation of NF-κB. (A) KF8 cells were pretreated with Celecoxib (25 μM) or DMSO (0.1%) for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for the indicated periods. Cytoplasmic extracts and nuclear extracts were prepared as described in Section 2 and immunoblotted with anti-p65 antibody, anti-c-jun antibody, anti-TRAF6 antibody or anti-lamin antibody (as a control). (B) KF8 cells were cultured on sterile coverslips and pretreated with Celecoxib (25 μM) or DMSO for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for 1 h. Cells were fixed in 4% paraformaldehyde and the localization of p65 was visualized with an antibody (green). DAPI (blue) was also applied to visualize nuclei.

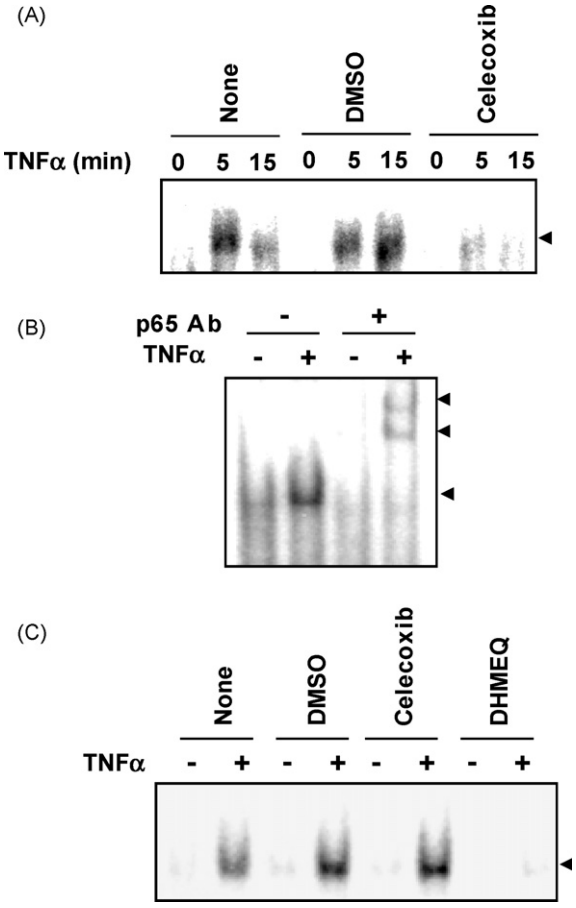

Fig. 5 – Celecoxib inhibited the TNFα-induced DNA binding activity of NF-κB. (A) KF8 cells were pretreated with Celecoxib (25 μM) or DMSO (0.1%) for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for the indicated periods. NF-κB DNA binding activity was measured by EMSA with a radiolabeled probe containing a consensus NF-κB binding site (5′-AGTTGAGGGGACTTTCCCAGG-3′). (B) For supershift assay, nuclear extracts were incubated in the presence of 1 μg anti-p65 antibody. (C) Celecoxib (25 μM) and DHMEQ (5 μg/mL) were added before incubation of the nuclear extract with radiolabeled NF-κB probe. *Arrows* denote specific NF-κB–DNA complexes.

after TNFα stimulation. To investigate the presence of cytoplasmic contamination, we evaluated the presence of TRAF6 and lamin in each sample by Western blotting analysis. TRAF6 and lamin have been reported to localize in cytoplasm and nucleus, respectively [28]. Little amount of TRAF6 was detected in nuclear extract, suggesting that the presence of cytoplasmic contamination was excluded in this assay. Furthermore, we performed immunofluoresence staining to investigate the effect of Celecoxib on the translocation of p65 NF-κB subunit. While NF-κB p65 was translocated into the nucleus after TNFα stimulation. Celecoxib clearly inhibited the TNFα-induced translocation of NF-κB p65. DMSO did not affect the nuclear localization of NF-κB p65 after TNFα stimulation (Fig. 4B). Moreover, we studied whether Celecoxib would also inhibit the TNFα-induced translocation of another transcription factor, c-jun; however, Celecoxib did not affect its nuclear translocation in response to TNFα (Fig. 4A). Thus, these results indicate that Celecoxib specifically inhibits nuclear translocation of NF-κB.

3.5. *Celecoxib inhibited the TNFα-induced DNA binding activity of NF-κB*

We next investigated the effect of Celecoxib on the DNA binding activity of NF-κB by EMSA using nuclear extract. EMSA indicated that TNFα induced rapid and marked NF-κB DNA binding activity, whereas Celecoxib significantly inhibited TNFα-induced activity (Fig. 5A). A supershift assay revealed that NF-κB DNA binding proteins are mostly p65, as shown in Fig. 5B. Furthermore, we also investigated whether Celecoxib has the ability to inhibit the DNA binding activity of NF-κB; however, when Celecoxib was added to the nuclear extract, it had no effect on the DNA binding activity of NF-κB (Fig. 5C). On the other hand, it was observed that dehydroxymethylepoxyquinomicin (DHMEQ), which has been shown to inhibit NF-κB activation at the level of DNA binding activity [29], clearly inhibited the DNA binding activity of NF-κB, when it was added to the nuclear extract treated with TNFα; therefore, it is suggested that Celecoxib should not inhibit the binding of NF-κB to the DNA directly (Fig. 5C).

3.6. *Celecoxib had no effect on TNFα-induced activation of MAP kinases and Akt*

It is well known that TNFα induces the MAP kinase family and Akt in various cells [30–32]. Furthermore, a previous study reported that activation of these kinases are important for the TNFα-induced expression of various inflammatory cytokines [30–32]. It was observed that ERK, JNK, p38 and Akt were significantly activated by TNFα; however, Celecoxib and DMSO had no effect on TNFα-induced activation of these kinases (Fig. 6). Thus, these data suggest that Celecoxib specifically inhibits TNFα-induced NF-κB activation.

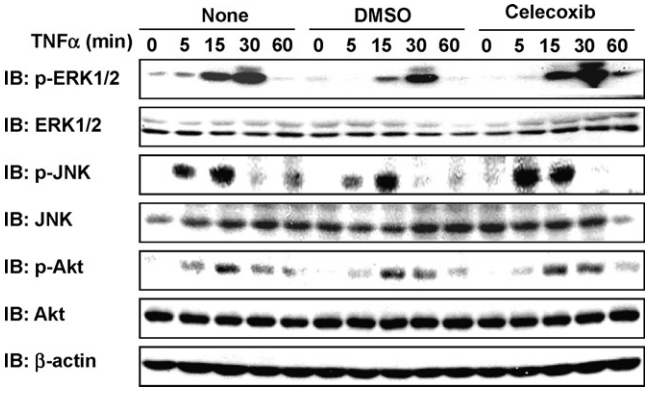

Fig. 6 – Celecoxib had no effect on TNFα-induced activation of MAP kinases and Akt. KF8 cells were pretreated with Celecoxib (25 μM) or DMSO (0.1%) for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for the indicated periods. Whole cell lysates were immunoblotted with antibodies recognizing phosphorylated ERK at Thr^{202}/Tyr^{204}, ERK, phosphorylated JNK at Thr^{183}/Tyr185, JNK, phosphorylated Akt at Ser^{473}, Akt, or β-actin.

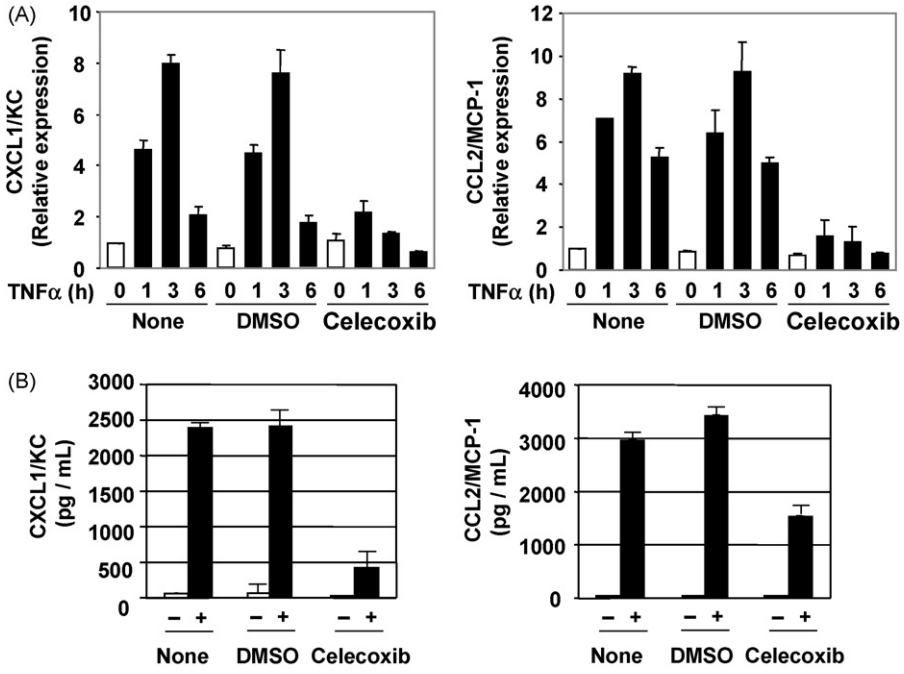

Fig. 7 – Celecoxib significantly inhibited TNFα-induced CXCL1/KC and CCL2/MCP-1 expressions. (A) KF8 cells were pretreated with Celecoxib (25 μM) or DMSO (0.1%) for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for the indicated periods. Total RNA was used to perform quantitative real-time RT-PCR with gene specific primers to CXCL1/KC and CCL2/MCP-1. Data are the mean ± S.D. of the relative expression levels in three experiments. (B) KF8 cells were pretreated with Celecoxib (25 μM) or DMSO for 1 h at 37 °C following stimulation with TNFα (10 ng/mL) for 16 h. CXCL1/KC or CCL2/MCP-1 in the cultured supernatants was measured with a commercial ELISA kit (R&D Systems). Data are shown as the mean ± S.D. of three independent experiments.

3.7. *Celecoxib significantly inhibited the TNFα-induced expression of inflammatory cytokines*

In order to examine whether the inhibition of NF-κB by Celecoxib could be translated to its inability to activate target genes such as CXCL1/KC and CCL2/MCP-1 in response to TNFα, quantitative real-time PCR was performed. When cells were stimulated with TNFα, marked expressions of CXCL1/KC and CCL2/MCP-1 mRNAs were induced at 1 h and detected until 3 h or 6 h, respectively. On the other hand, treatment with Celecoxib significantly inhibited the TNFα-induced expressions of CXCL1/KC and CCL2/MCP-1 (Fig. 7A). Consistently, Celecoxib potently reduced the TNFα-induced secretion of CXCL1/KC and CCL2/MCP-1, as shown in Fig. 7B. These data suggest that Celecoxib shows anti-inflammatory activity by inhibiting the expression of various TNFα-induced inflammatory cytokines.

4. Discussion

Celecoxib is in a class of NSAIDs called COX2 inhibitors, which binds the catalytic domain of COX2 [7]. It is used to relieve pain, tenderness, swelling and stiffness caused by osteoarthritis, rheumatoid arthritis and ankylosing spondylitis [8–10]; however, the detailed anti-inflammatory activity of Celecoxib remains to be explored. In this study, we demonstrated that TNFα-induced NF-κB activation and subsequent CXCL1/KC and CCL2/MCP-1 gene activations were severely inhibited by Celecoxib. RA is an autoinflammatory disorder in which TNFα plays an important role leading to the excessive production of various inflammatory cytokines [11–13]. Therefore, this inhibitory effect on TNFα-induced cytokine expressions suggests one of the anti-inflammatory actions of Celecoxib in the treatment of RA. Moreover, these results might be very interesting to exhibit the COX2-independent anti-inflammatory effect of Celecoxib.

Previously, Shishodia et al. reported that Celecoxib suppressed NF-κB activation induced by various stimulations, including TNFα, phorbol ester, okadaic acid, LPS, and IL-1β [33]; however, it should be noted that a much higher concentration of Celecoxib was used in their study to inhibit NF-κB activation than in our study. In their study, 100 μM of Celecoxib was used to manifest the inhibition of TNFα-induced IKK activation, leading to the suppression of IκBα phosphorylation and degradation. However, using 100 μM of Celecoxib, the inhibition of MAP kinase family and severe cytotoxity were also observed, suggesting that high concentration of Celecoxib exhibits various effects rather than the inhibition of NF-κB [33–37]. To address the specific effect of Celecoxib on inflammatory responses, we utilized 25 μM of Celecoxib, with which NF-κB activation effectively inhibited as shown in Fig. 1. Celecoxib had no effect on TNFα-induced JNK, p38, ERK and Akt activation under little cytotoxicity (Figs. 1C

and 6). Additionally, 25 μM of Celecoxib failed to inhibit TNFα-induced IKK activation and degradation of IκBα and IκBβ, although this concentration of Celecoxib is enough to inhibit the expression of NF-κB target genes. Interestingly, since 25 μM of Celecoxib effectively suppressed the nuclear translocation of NF-κB, this could be the mechanism how lower concentration of Celecoxib inhibited NF-κB activation. The detailed mechanism how Celecoxib specifically suppress the nuclear translocation of NF-κB still remains unclear. A clue to resolve this problem is our result showing Celecoxib failed to suppress nuclear translocation of c-jun. The difference of translocation mechanism between NF-κB and c-jun has been reported. Importinα3 and α4 accelerate the nuclear translocation of NF-κB, however, importinα family oppositely inhibits the nuclear translocation of c-jun [38,39]. Although we have no additional data, the difference of importin usage may define the specificity of Celecoxib's effect. As another possibility, Celecoxib might directly bind to NF-κB and suppress the function of its nuclear localization signal (NLS). This model is simple to explain why Celecoxib specifically inhibit nuclear translocation of NF-κB. To clarify this problem, it is required to test if Celecoxib directly binds to NF-κB or other molecules involved in NF-κB nuclear localization.

In the recent studies, it has also been well established that phosphorylations of NF-κB p65 subunit, at Ser 276 and Ser 536, which are curried out by protein kinase A and IKKα, respectively, are important for the regulation of NF-κB [40–43]. Interestingly, Celecoxib failed to affect these phosphorylations, suggesting that these phosphorylations have to be upstream of NF-κB nuclear translocation (data not shown). It is also reported that another selective COX2 inhibitor named Rofecoxib is also known to inhibit the activation of NF-κB [44]. Callejas et al. reported that Rofecoxib suppressed NF-κB activation via inhibiting IKK activation in murine macrophages treated with lipopolysaccharide [44]. However, Callejas et al. also utilized high concentration of Rofecoxib and this compound harbors a quite similar structure with Celecoxib, suggesting that usage of Rofecoxib with lower concentration may display more specific effect. The study about these similar compounds would provide us a clue to develop the more specific therapeutic drug against inflammatory diseases.

Dehydroxymethylepoxyquinomicin has been shown to inhibit NF-κB activation via suppression of nuclear translocation and DNA binding activity [29]. This compound had no effect on TNFα-induced IKK activation and IκBα degradation, suggesting that Celecoxib and DHMEQ may inhibit the activation of NF-κB with similar mechanism. However, whereas DHMEQ inhibited the DNA binding activity of NF-κB, Celecoxib did not inhibit its DNA binding activity when Celecoxib was added before incubation of the nuclear extract with DNA (Fig. 5C). We have no explanation about this difference. However, it could be speculated that DHMEQ may inhibit NF-κB nuclear translocation via the direct interaction with NF-κB causing the severe structural change of NF-κB, whereas Celecoxib may indirectly disturb NF-κB activation via inhibiting the components required for nuclear translocation of NF-κB.

The number of NF-κB functions has been rapidly increasing; for example, NF-κB activation is important in not only the inflammatory reaction and immune responses but also anti-apoptotic functions and cell cycle control [45,46]. NF-κB is often activated in various tumors, including breast cancer cells and carcinoma cells [47,48]. Furthermore, it was also reported that inactivation of NF-κB can sensitize cancer cells to the effects of chemotherapy [49], suggesting that Celecoxib would be useful as anti-cancer drug.

In this study, we showed that Celecoxib inhibited NF-κB activation through a unique mechanism, compared with other NF-κB inhibitors. Therefore, further investigating the mechanism of how Celecoxib negatively regulates TNFα-induced NF-κB activation is expected to show not only its novel anti-inflammatory mechanism but also to provide a precise understanding of the NF-κB activation mechanism.

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